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(54) Title: HUMAN CERBERUS PROTEIN

(57) Abstract

Human Cerberus proteins and related nucleic acids are provided. Included are proteins comprising a human cerberus domain having specific activity, particularly the ability to antagonize a bone morphogenic protein. The proteins may be produced recombinantly from transformed host cells with the subject nucleic acids. Also provided are isolated hybridization probes and primers capable of specifically hybridizing with the disclosed genes, specific binding agents and methods of making and using the subject compositions.

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HUMAN CERBERUS PROTEIN

This International Application claims priority of U.S. Provisional Application
5 60/045,157 filed April 30, 1997 and U.S. Provisional Application 60/044,427 filed
April 29, 1997. All publications, patents and patent applications cited in this
specification are hereby incorporated by reference as if each individual
publication, patent or patent application was specifically and individually
indicated to be incorporated by reference.

10

INTRODUCTIONField of the Invention

The field of this invention is proteins which regulate cell function, and in
15 particular, induce neural development or antagonize bone morphogenic
proteins.

Background

Natural regulators of cellular growth, differentiation and function have
provided important pharmaceuticals, clinical and laboratory tools, and targets for
therapeutic intervention. A variety of such regulators have been shown to have
profound effects on basic cellular differentiation and developmental pathways.
For example, the recently cloned Xenopus cerberus protein induces the
25 formation of head structures in anterior endoderm of vertebrate embryos.
Similarly, the noggin protein induces head structures in vertebrate embryos, and
can redirect mesodermal fates from ventral fates, such as blood and
mesenchyme, to dorsal fates such as muscle and notochord and can redirect
epidermal fates to anterior neural fates. The activities of chordin are similar to
30 those of noggin, reflecting a common mechanism of action - namely
antagonizing bone morphogenic proteins (BMP) and thereby preventing their
function. BMPs have diverse biological activities in different biological contexts,

including the induction of cartilage, bone and connective tissue, and roles in kidney, tooth, gut, skin and hair development.

Different members of the TGF β superfamily can instruct cells to follow different

5 fates, for example TGF β induces neural crest to form smooth muscle, while BMP2 induces the same cells to become neurons. In *Xenopus* experiments, dissociated animal cap cells (prospective ectoderm) become epidermis in response to BMP4 but become mesoderm in response to activin.

10 Since the sequence identity between activin and BMP4 is low, it is not surprising that they induce different fates. It is more surprising that members of the BMP subfamily, which are quite closely related in sequence, can induce distinct fates. A striking example results from implantation of a matrix impregnated with a BMP into muscle; when the effects are monitored histologically, BMP2, 4 and 7

15 induce endochondral bone formation, whereas a related molecule BMP12/GDF7 induces connective tissue similar to tendon. Similarly, BMP4 can induce cell death in the hindbrain neural crest, while the related protein dorsalin does not.

Since different BMP family members can induce different fates, then BMP antagonists that have specificity in blocking subsets of BMPs could change the balance of BMPs that are presented to a cell, thus altering cell fate. In view of the importance of relative BMP expression in human health and disease, regulators of cellular function and BMP function in particular, such as noggin and cerberus, provide valuable reagents with a host of clinical and biotechnological applications. The present invention relates to a new family of regulators of cellular function.

Relevant Literature

30

Bouwmeester, T., et al. (1996) Nature 382: 595-601 describe cloning of the *Xenopus*

cerberus gene and provide a deduced amino acid sequence of *Xenopus* cerberus protein in addition to describing its biological activity. Lamb, T. M., et al. (1993) Science 262: 713-718; Smith, W. C., et al. (1992) Cell 70: 829-840; Smith, W. C., et al. (1993) Nature 361: 547-549; and Zimmerman, L. B., et al. (1996) Cell 86: 599-606
5 describe the isolation and function of the noggin protein. Piccolo, S., et al. (1996) Cell 86: 589-598, Sasai, Y., et al. (1995) Nature 376: 333-336, and Sasai, Y., et al.
(1994) Cell 79: 779-790 relate to the chordin protein. Enomoto et al. (1994)
Oncogene 9: 2785-2791 and Ozaki, et al. (1996) Jpn. J. Cancer Res. 87: 58-61 describe
human and murine homologs of the DAN gene.

10

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to human cerberus protein and related nucleic acids. Included are proteins comprising a human
15 cerberus domain and having human cerberus-specific activity. The proteins may be produced recombinantly from transformed host cells with the subject nucleic acids. The invention provides binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g., genetic hybridization screens for human cerberus transcripts), therapy (e.g., gene therapy
20 to modulate human cerberus gene expression) and in the biopharmaceutical industry (e.g., reagents for screening chemical libraries for lead pharmacological agents).

Preferred uses for the subject human cerberus proteins include modifying the
25 physiology of a cell comprising an extracellular surface by contacting the cell or medium surrounding the cell with an exogenous human cerberus protein under conditions whereby the added protein specifically interacts with a component of the medium and/or the extracellular surface to effect a change in the physiology of the cell. Also preferred are methods for screening for biologically active
30 agents, which methods involve incubating a human cerberus protein in the presence of an extracellular human cerberus protein-specific binding target and a candidate agent,

under conditions whereby, but for the presence of the agent, the protein specifically binds the binding target at a reference affinity; detecting the binding affinity of the protein to the binding target to determine an agent-biased affinity, wherein a difference between the agent-biased affinity and the reference affinity
5 indicates that the agent modulates the binding of the protein to the binding target.

BRIEF DESCRIPTION OF THE FIGURE

10 FIGURE 1 - Western Blot showing binding of human cerberus to human BMP2 in lane 1. Addition of a human noggin deletion mutein blocks binding of human cerberus to human BMP2 as shown in lane 2. Control for non-specific binding shown in lane 3.

15 DETAILED DESCRIPTION OF THE INVENTION

The invention provides human cerberus protein which includes natural human cerberus protein and recombinant proteins comprising a human cerberus amino acid sequence, or a functional human cerberus protein domain thereof having an
20 assay-discernable human cerberus-specific activity. Accordingly, the proteins may be deletion mutants of the disclosed natural human cerberus proteins and may be provided as fusion products, e.g., with non-human cerberus polypeptides. The subject human cerberus protein domains have human cerberus-specific activity or function and are functionally distinct from each other and from b57,
25 DAN and noggin.

Without being bound by theory, we have formulated hypotheses about the embryological effects of cerberus based on where it is expressed, and on the effects of RNA injection in embryos. Since cerberus is expressed in the Spemann
30 organizer, we believe cerberus to be a mediator of some of the effects of the Spemann organizer, such as neural induction. Since cerberus is expressed in regions that will become heart primordium, we believe cerberus to influence

either the dorsal-ventral pattern or anterior-posterior pattern of the neural plate. In the frog, cerberus occupies the anteriormost endomesoderm. This localized expression of cerberus in the frog may give rise to heart primordium. Although cerberus is unable to dorsalize ventral mesoderm and does not rescue axis

5 formation in ventralized frog embryos, it has been known to promote formation of anterior neural structures such as cement gland, brain, and olfactory placodes. Cerberus exhibits homology to b57, a protein that has been shown to directly bind BMP-2 and BMP-4 and inhibit their biological actions. This effect has also been shown to be mediated by the unrelated protein noggin which is also expressed in
10 the Spemann organizer and which has been shown to induce neural ectoderm. By inference, cerberus may also be a direct inhibitor of BMP activity, and this may account for its known biological effects.

A number of applications for cerberus are suggested from its properties.

15 Cerberus, like noggin and b57, may be useful in the study and treatment of heart disease and neurological disorders, as well as pathological conditions that arise from or involve heterotopic bone formation, cartilage or cartilagenous plaques. Furthermore, the cerberus cDNA may be useful as a diagnostic tool, such as through use of antibodies in assays for proteins in cell lines or use of
20 oligonucleotides as primers in a PCR test to amplify those with sequence similarities to the oligonucleotide primer, and to see how much cerberus is present. The isolation of human cerberus, of course, also provides the key to isolate its putative receptor, other cerberus binding proteins, and/or study its antagonistic properties.

25 Human cerberus-specific activity or function may be determined by convenient in vitro, cell-based, or in vivo assays - e.g., in vitro binding assays, cell culture assays, in animals (e.g., immune response, gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the specific molecular interaction of a
30 human cerberus protein with a binding target is evaluated. The binding target may be a natural binding target, or a non-natural binding target such as a specific immune protein such as an antibody, or a human cerberus specific agent such as

those identified in assays described below. Generally, binding specificity is assayed by bioassay (e.g., the ability to induce neuronal tissue from injected embryonic ectoderm), TGF β protein binding equilibrium constants (usually at least about 10^7 M $^{-1}$, preferably at least about 10^8 M $^{-1}$, more preferably at least about 5 10 9 M $^{-1}$), by the ability of the subject protein to function as negative mutants in human cerberus-expressing cells, to elicit human cerberus specific antibody in a heterologous host (e.g., a rodent or rabbit), etc.

The claimed proteins may be isolated or pure - an "isolated" protein is one that is 10 no longer accompanied by some of the material with which it is associated in its natural state, and that preferably constitutes at least about 0.5%, and more preferably at least about 5% by weight of the total protein in a given sample; a "pure" protein constitutes at least about 90%, and preferably at least about 99% by weight of the total protein in a given sample. The subject proteins and protein 15 domains may be synthesized, produced by recombinant technology, or purified from cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. 20 Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY). An exemplary method for isolating natural human cerberus protein involves expressing a cDNA library (e.g., one derived from Xenopus ovarian cells) and assaying expression products for embryonic axis formation. This method and other suitable bioassays amenable to detecting human cerberus proteins have been 25 described by Lemaire, P., et al., (1995) Cell 81:85-94; Smith, W. C., and Harland, R. M. (1992) Cell 70: 829-40; Smith, W. C., and Harland, R. M. (1991) Cell 67: 753-765; Piccolo, S., et al., (1996) Cell 86: 589-98; and Zimmerman, L. B., et al., (1996) Cell 86: 599-606.

30 The subject proteins find a wide variety of uses including use as immunogens, targets in screening assays, bioactive reagents for modulating cell growth,

differentiation and/or function, etc. For example, the invention provides methods for modifying the physiology of a cell comprising an extracellular surface by contacting the cell or medium surrounding the cell with an exogenous human cerberus protein under conditions whereby the added protein specifically

5 interacts with a component of the medium and/or the extracellular surface to effect a change in the physiology of the cell. According to these methods, the extracellular surface includes plasma membrane-associated receptors; the exogenous human cerberus refers to a protein not made by the cell or, if so, expressed at non-natural levels, times or physiologic locales; and suitable media

10 include in vitro culture media and physiological fluids such as blood, synovial fluid, etc. Effective administrations of subject proteins may be used to reduce undesirable (e.g., ectopic) bone formation, inhibit the growth of cells that require a morphogenic protein (e.g., BMP-dependent neuroblastomas and gliomas), alter morphogen-dependent cell fate/differentiation in culture, such as with cells for

15 transplantation or infusion, etc. The proteins may be introduced, expressed, or repressed in specific populations of cells by any convenient way such as microinjection, promoter-specific expression of recombinant enzyme, targeted delivery of lipid vesicles, etc.

20 The invention provides natural and non-natural human cerberus-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. Human cerberus-specific binding agents include human cerberus-specific receptors, such as somatically recombined protein receptors like specific antibodies or T-cell antigen receptors

25 (See, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and also includes other natural binding agents identified with assays such as one-, two- and three-hybrid screens, and non-natural binding agents identified in screens of chemical libraries such as described below. Agents of particular interest modulate human cerberus function.

30 The invention provides human cerberus nucleic acids, which find a wide variety of applications including use as translatable transcripts, hybridization probes,

PCR primers, diagnostic nucleic acids, etc., as well as use in detecting the presence of human cerberus genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional human cerberus homologs and structural analogs.

5

The subject nucleic acids are of synthetic/non-natural sequences and/or are isolated, i.e., no longer accompanied by some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of total nucleic acid present in a given fraction, and usually recombinant, meaning they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome. Nucleic acids comprising the nucleotide sequence disclosed herein and fragments thereof, contain such sequence or fragment at a terminus, immediately flanked by a sequence other than that to which it is joined on a natural chromosome, or flanked by a native flanking region fewer than 10 kb, preferably fewer than 2 kb, which is immediately flanked by a sequence other than that to which it is joined on a natural chromosome. While the nucleic acids are usually RNA or DNA, it is often advantageous to use nucleic acids comprising other bases or nucleotide analogs to provide modified stability, etc.

The amino acid sequences of the disclosed human cerberus protein is used to back translate human cerberus protein-encoding nucleic acids optimized for selected expression systems (Holler, et al. (1993) Gene 136: 323-328; Martin, et al. (1995) Gene 154: 150-166) or used to generate degenerate oligonucleotide primers and probes for use in the isolation of natural human cerberus encoding nucleic acid sequences ("GCG" software, Genetics Computer Group, Inc., Madison, WI). Human cerberus encoding nucleic acids may be part of expression vectors and may be incorporated into recombinant host cells, e.g., for expression and screening, for transgenic animals, for functional studies such as the efficacy of candidate drugs for disease associated with human cerberus mediated signal transduction, etc. Expression systems are selected and/or tailored to effect

human cerberus protein structural and functional variants through alternative post-translational processing.

The invention also provides for nucleic acid hybridization probes and

5 replication/amplification primers having a human cerberus cDNA specific sequence and sufficient to effect specific hybridization with SEQ. NO. 1.

Demonstrating specific hybridization generally requires stringent conditions, for example, hybridizing in a buffer comprising 30% formamide in 5 x SSPE (0.18 M NaCl, 0.01 M NaPO₄, pH7.7, 0.001 M EDTA) buffer at a temperature of 42°C and

10 remaining bound when subject to washing at 42°C with 0.2 x SSPE; preferably hybridizing in a buffer comprising 50% formamide in 5 x SSPE buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2x SSPE buffer at 42°C. Human cerberus cDNA homologs can also be distinguished from other protein using alignment algorithms, such as BLASTX
15 (Altschul, et al. (1990) Basic Local Alignment Search Tool, J. Mol. Biol. 215: 403-410).

Human cerberus hybridization probes find use in identifying wild-type and mutant alleles in clinical and laboratory samples. Mutant alleles are used to

20 generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. Human cerberus nucleic acids are also used to modulate cellular expression or intracellular concentration or availability of active human cerberus. Human cerberus inhibitory nucleic acids are typically antisense - single stranded sequences comprising complements of the disclosed natural human
25 cerberus coding sequences. Antisense modulation of the expression of a given human cerberus protein may employ antisense nucleic acids operably linked to gene regulatory sequences. Cells are transfected with a vector comprising a human cerberus sequence with a promoter sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to
30 endogenous human cerberus encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for

stable extrachromosomal maintenance or integration. Alternatively, single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a given human cerberus protein may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial

5 reduction in expression of the targeted protein. An enhancement in human cerberus expression is effected by introducing into the targeted cell type human cerberus nucleic acids which increase the functional expression of the corresponding gene products. Such nucleic acids may be human cerberus expression vectors, vectors which upregulate the functional expression of an
10 endogenous allele, or replacement vectors for targeted correction of mutant alleles. Techniques for introducing the nucleic acids into viable cells are known in the art and include retroviral-based transfection, viral coat protein-liposome mediated transfection, etc.

15 The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of human cerberus modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate human cerberus interaction with a natural human cerberus binding target. A wide variety of assays for binding agents are provided
20 including protein-protein binding assays, immunoassays, cell based assays, etc. Preferred methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds.

In vitro binding assays employ a mixture of components including a human

25 cerberus protein, which may be part of a fusion product with another peptide or polypeptide, e.g., a tag for detection or anchoring, etc. The assay mixtures comprise a natural human cerberus binding target. While native binding targets may be used, it is frequently preferred to use portions thereof as long as the portion provides binding affinity and avidity to the subject human cerberus
30 conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds, preferably small

organic compounds, and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents such as salts, buffers, neutral proteins, e.g., albumin, detergents, protease inhibitors, nucleic acid inhibitors, antimicrobial agents, etc., may also be included. The mixture
5 components can be added in any order that provides for the requisite bindings and incubations may be performed at any temperature which facilitates optimal binding. The mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the human cerberus specifically binds the cellular binding target, portion or analog with a reference binding
10 affinity. Incubation periods are chosen for optimal binding but are also minimized to facilitate rapid, high throughput screening.

After incubation, the agent-biased binding between the human cerberus and one or more binding targets is detected by any convenient way. For cell-free binding
15 type assays, a separation step is often used to separate bound from unbound components. Separation may be effected by precipitation, immobilization, etc., followed by washing by, e.g., membrane filtration or gel chromatography. For cell-free binding assays, one of the components usually comprises or is coupled to a label. The label may provide for direct detection as radioactivity, luminescence,
20 optical or electron density, etc., or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g., through optical or electron density, radiative emissions, nonradiative energy transfers, or indirectly detected with antibody conjugates, etc. A difference in the binding affinity of the
25 human cerberus protein to the target in the absence of the agent as compared with the binding affinity in the presence of the agent indicates that the agent modulates the binding of the human cerberus protein to the corresponding binding target. A difference, as used herein, is statistically significant and preferably represents at least a 50%, more preferably at least a 90% difference.

30

The invention provides for a method for modifying the physiology of a cell comprising an extracellular surface in contact with a medium, said method

comprising the step of contacting said medium with an exogenous human cerberus protein under conditions whereby said protein specifically interacts with at least one of a component of said medium and said extracellular surface to effect a change in the physiology of said cell.

5

The invention further provides for a method for screening for biologically active agents, said method comprising the steps of a) incubating a human cerberus protein in the presence of an extracellular human cerberus protein specific binding target and a candidate agent, under conditions whereby, but for the 10 presence of said agent, said protein specifically binds said binding target at a reference affinity; b) detecting the binding affinity of said protein to said binding target to determine an agent-biased affinity, wherein a difference between the agent-biased affinity and the reference affinity indicates that said agent modulates the binding of said protein to said binding target.

15

One embodiment of the invention is an isolated human cerberus protein comprising the amino acid sequence as set forth herein or a fragment thereof having human cerberus-specific activity.

20 Another embodiment of the invention is a recombinant nucleic acid encoding human cerberus protein comprising the amino acid sequence as set forth herein or a fragment thereof having human cerberus-specific activity.

25 Still another embodiment is an isolated nucleic acid comprising a nucleotide sequence as set forth herein or a fragment thereof having at least 18 consecutive bases and sufficient to specifically hybridize with a nucleic acid having the sequence of set forth herein in the presence of natural human cerberus cDNA.

Applicants have devised a method of screening for a molecule capable of 30 competing with human cerberus for binding to a BMP comprising:
a) contacting a sample suspected of containing the molecule with the BMP in the presence of human cerberus under conditions in which the human cerberus is

capable of binding to the BMP; and b) detecting binding of the molecule to the BMP.

In a preferred embodiment, the human cerberus is detectably labeled and includes, but is not limited to, human cerberus linked covalently or noncovalently to a radioactive substance, a fluorescent substance, a substance that has enzymatic activity, a substance that may serve as a substrate for an enzyme (enzymes and substrates associated with colorimetrically detectable reactions are preferred) or to a substance that can be recognized by an antibody molecule that is preferably a detectably labeled antibody molecule.

By way of nonlimiting example, the method may be performed via an assay which is conceptually similar to an ELISA assay. For example, a BMP may be bound to a solid support, such as a plastic multiwell plate. As a control, a known amount of a molecule which contains the human cerberus BMP-binding domain and which has been Myc-tagged may then be introduced to the well and any tagged molecules which bind the BMP may then be identified by means of a reporter antibody directed against the Myc-tag. This assay system may then be used to screen test samples for molecules which are capable of i) binding to the tagged molecule or ii) binding to the BMP and thereby blocking binding to the BMP by the tagged molecule. For example, a test sample containing a putative molecule of interest together with a known amount of a tagged molecule which contains the human cerberus BMP-binding domain may be introduced to the well and the amount of tagged molecule which binds to the BMP may be measured. By comparing the amount of bound tagged molecule in the test well to the amount in the control well, samples containing molecules which are capable of blocking tagged molecule binding to the BMP may be identified. The molecules of interest thus identified may be isolated using methods well known to one of skill in the art.

30

Once a blocker of BMP binding is found, one of skill in the art would know to perform secondary assays to determine whether the blocker is binding to the

tagged molecule or to the BMP, as well as assays to determine if the blocker molecule can neutralize the biological activity of the bound molecule.

The present invention also provides for antibodies to the human cerberus protein described herein which are useful for detection of the protein in, for example, diagnostic applications. For preparation of monoclonal antibodies directed toward this human cerberus protein, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

15 The monoclonal antibodies for diagnostic or therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:7308-7312; Kozbor et al., 1983, *Immunology Today* 4:72-79; Olsson et al., 1982, *Meth. Enzymol.* 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851, Takeda et al., 1985, *Nature* 314:452).

25 Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of the human cerberus protein described herein. For the production of antibody, various host animals can be immunized by injection with the human cerberus protein, or a fragment or derivative thereof, 30 including but not limited to rabbits, mice and rats. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels

such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Corynebacterium parvum.

5

A molecular clone of an antibody to a selected human cerberus protein epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct 10 nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

The present invention provides for antibody molecules as well as fragments of such antibody molecules. Antibody fragments which contain the idiotype of the 15 molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain 20 and a reducing agent. Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof.

25 The invention further provides for a method of using a human cerberus protein or fragment thereof as an antagonist of the activity of a bone morphogenic protein (BMP). Preferably, the invention provides for a method of antagonizing the function of a Bone Morphogenic Protein (BMP) which comprises contacting said BMP with human cerberus protein or a fragment thereof. The method of 30 the invention is carried out under conditions whereby the human cerberus or fragment thereof binds to the BMP. In further preferred embodiments of the

invention, the human cerberus or fragment thereof is used to antagonize the function of BMP2 or BMP4.

Antagonists to BMPs may be useful for preventing and treating BMP-related disorders of animals, especially of humans. It was, therefore, an object of this invention to identify substances which effectively antagonize the function of BMPs in disease states in animals, preferably mammals, especially in humans. It was another object of this invention to prepare novel compounds which inhibit BMP. It was still another object of this invention to develop a method of antagonizing the functions of BMPs in disease states in mammals. It was also an object of this invention to develop a method of preventing or treating disorders relating to the function of BMPs.

In addition to their roles in normal bone formation, the BMPs appear to be involved in diseases in which they promote abnormal bone growth. For example, BMPs have been reported to play a causative role in the disease known as Fibrodysplasia Ossificans Progressiva (FOP), in which patients grow an abnormal "second skeleton" that prevents any movement.

Therefore, an object of the present invention is to provide a novel molecule for the treatment of diseases or disorders including, but not limited to, Fibrodysplasia Ossificans Progressiva (FOP). Since human cerberus binds BMPs, it offers hope as a therapeutic agent for this disease. Additionally, abnormal bone growth can occur after hip replacement surgery and thus ruin the surgical outcome. This is a more common example of pathological bone growth and a situation in which binders of BMPs such as human cerberus may be therapeutically useful. Human cerberus may also be useful for treating other forms of abnormal bone growth, such as the pathological growth of bone following trauma, burns or spinal cord injury. In addition, human cerberus may be useful for treating or preventing the undesirable actions of BMPs associated with the abnormal bone growth seen in connection with metastatic prostate cancer or osteosarcoma.

In additional embodiments, the human cerberus nucleic acids, proteins, fragments and peptides of the invention may be used to antagonize BMP activity in mammals.

5

The present invention also provides for compositions comprising a human cerberus molecule, as described herein and a suitable carrier. The active ingredient, which may comprise the human cerberus, should be formulated in a suitable carrier for systemic or local administration in vivo by any appropriate route including, but not limited to injection (e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, endoneurial, perineural, intraspinal, intraventricular, intravitreal, intrathecal etc.), by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.); or by a sustained release implant, including a cellular or tissue implant.

15

Depending upon the mode of administration, the active ingredient may be formulated in a liquid carrier such as saline, incorporated into liposomes, microcapsules, polymer or wax-based and controlled release preparations, or formulated into tablet, pill or capsule forms.

20

The concentration of the active ingredient used in the formulation will depend upon the effective dose required and the mode of administration used. The dose used should be sufficient to achieve circulating plasma concentrations of active ingredient that are efficacious. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

The following examples are offered by way of illustration and not by way of limitation.

Example 1 - Cloning and Sequencing of Human cerberus gene

A human genomic library (Genome Systems, Inc. - Human release II BAC-4435) was hybridized to a mouse probe approximately 314 nucleotides in length that

5 was obtained by PCR from a mouse EST clone (GenBank accession number AA120122; clone number 538769). DNA from human clones hybridizing to the mouse probe was digested with restriction enzymes, Southern blotted and hybridized. Those DNA fragments hybridizing to the mouse probe were purified, then subcloned using a commercial cloning kit (Zero Blunt PCR

10 Cloning Kit, Invitrogen Cat# K2700-20) and sequenced using the ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence obtained corresponded to a segment of human cerberus encoding a polypeptide starting from the amino acid Threonine at position 170 (see SEQ ID No. 2) to a point beyond the stop codon.

15 The RACE procedure was then used to obtain the full length human cerberus nucleotide sequence as follows. Oligonucleotides were designed based on the partial human sequence and used as primers for the reverse transcriptase reaction and for PCR. An approximately 1.2 Kb fragment was isolated and sequenced and found to contain the remainder of the human cerberus sequence.

20 The sequence of human cerberus (SEQ ID NO. 1) was then further confirmed by PCR and by sequencing of a full length cerberus cDNA and also by partial direct sequencing of the human genomic clone described above.

25 Example 2 - Construction of human Cerberus (hCer) expression plasmid pRG629

A DNA fragment encoding the gene for hCerberus was PCR amplified from pMT21.hCer.Fc using the primers N1-hCer (5'-AACATGATGCAGGATGG
CCGCCAG-3') and C1-hCer (5'-GAGAGCGGCCGCTCATTAAGCTGAAACT
30 CCTGGGATAAAGGAATCCTGGG-3'). The resulting 769 bp fragment was digested with Not 1 then ligated into the Pme 1-Not 1 sites of pRG461 a high copy vector encoding the gene for kanamycin resistance. This vector contains the

phage T7 Φ 1.1 promoter which directs the transcription of genes inserted into the Pme1 site. pRG461 was constructed at Regeneron. A clone was identified and named pRG629, the construct was confirmed by DNA sequence analysis, then transformed into *E. coli* strain RFJ143 by electroporation. RFJ143 is an *E. coli* strain constructed at Regeneron and is essentially *E. coli* K12 strain W3110 lacIQ ZpL8 Z+ Y+ fhuA Δ 322-405 ara (DE3).

Example 3 - Purification of human Cerberus protein

10 *E. coli* strain RFJ143 containing pRG629 was grown in LB medium (Difco) and expression of hCerberus was induced by the addition of 1 mM IPTG. Induced cells were collected by centrifugation, resuspended in 10 volumes of 100 mM Tris-HCl, pH 8.5, 20 mM EDTA, and lysed by passage through a Niro-Soave Panda cell disrupter (Niro-Soave) to release inclusion bodies. The cell lysate was
15 centrifuged and the pellet was resuspended in 10 volumes of 6 M guanidinium-HCl, 100 mM Tris-HCl, pH 8.5, 10 mM EDTA, 100 mM Na₂SO₃, 10 mM Na₂S₄O₆ and stirred for 16 hr at room temperature. The solubilized inclusion bodies were fractionated on a Sephadryl S-300 column (Pharmacia) equilibrated in 8 M urea,
20 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA. Fractions containing hCerberus were pooled, diluted with 4 volumes of 6 M urea, 20 mM MES (2-(N-Morpholino)ethanesulfonic acid), pH 6.0 then loaded onto an SP-Sepharose (Pharmacia) column equilibrated with 6 M urea, 20 mM MES, pH 6.0 and eluted from the column with a linear gradient of NaCl in 6 M urea, 20 mM MES, pH 6.0. Purified cerberus was refolded by dilution with 10 volumes of buffer to 3.5 M
25 urea, 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 0.5 mM cysteine followed by incubation at 4°C. After 2 days incubation, the refold mix was loaded onto a Q-Sepharose column (Pharmacia) equilibrated with 4 M urea, 50 mM Tris-HCl, pH 9.5, 0.1 mM EDTA, 20% glycerol ad eluted with a linear NaCl gradient in the same buffer. Fractions containing hCerberus were pooled and dialyzed against 20
30 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA. The dialysate was acidified with 0.1 % TFA, loaded onto a Jupiter C5 column (Phenomenex) equilibrated in

0.1% TFA, 10% acetonitrile, and eluted with an increasing acetonitrile gradient from 30% to 50% at 1.3%/min. Fractions containing hCerberus were pooled, dried under vacuum, then resuspended in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA.

5

Example 4 - Demonstration That Human Cerberus Binds to Human BMP2

Human Cerberus (1 µg/ml) that was expressed in E. coli and refolded was co-incubated with hBMP2 (1 µg/ml) in the absence or in the presence of human noggin protein (hNGΔB2, 2 µg/ml). Human noggin binds to BMP2 with high affinity. The hNGΔB2 is a deletion mutein of human noggin that displays identical biological activity to human noggin but has reduced binding to heparin. Thus addition of noggin (hNGΔB2) should inhibit binding of hCER to BMP2, if an excess of noggin is added over hCER and BMP2.

15

The formation of a stable complex between hCER and the BMPs was determined by immunoprecipitating hCER and associated proteins using an anti-hCER antisera bound to Protein G-Sepharose beads (Pharmacia). The binding reaction was carried out in binding buffer, which is comprised of 20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween 20 (TBST), 1 mg/ml bovine serum albumin (BSA).

Binding was allowed to proceed for 1 hour, at 25°C, in a reaction volume of 1 ml, with continuous mixing to keep the Protein G-Sepharose in suspension, after which point the beads were spun down, washed once with TBST, moved to new eppendorf tubes, and washed 3 more times with TBST. Proteins bound to the beads were solubilized by addition of 25 µl of Laemmli SDS-PAGE sample buffer (see Sambrook, et al. - A Cloning Manual, Cold Spring Harbor Laboratory) and loaded onto 4 to 12% NuPAGE/MES gradient gels (Novex), which were run under reducing conditions. The proteins were subsequently transferred on Immobilon P (Millipore) and Western blotted for the presence of BMP2 or BMP4 using polyclonal antisera raised against the respective proteins.

As can be seen in Figure 1, hCER binds to hBMP2 (lane 1). Addition of hNGΔB2

blocks this interaction (lane 2), by binding to hBMP2 and blocking its ability to bind to hCER. This indicates that the epitope recognized by hCER on hBMP2 is the same or overlaps with the epitope recognized by noggin, or alternatively that binding of noggin to BMP2 and BMP4 sterically hinders the binding of hCER.

5 There was no binding of hBMP2 to the beads if hCER was omitted from the reaction (lane 3), indicating that there is no non-specific binding of hBMP2 to the beads and that the observed binding is hCER-dependent. It should be noted that identical results have been obtained when examining the interaction of hCER with BMP4 and also when using different tagged forms of hCER, such as hCER-
10 FLAG, hCER-myc and hCER-Fc. The tagged forms may be produced using standard genetic engineering techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY)).

15

Example 5 - Tissue Expression of Human Cerberus

We have examined the expression of human Cerberus by analysis of polyA+ mRNA prepared from different adult human tissues. TABLE 1 lists the tissues
20 tested and the relative level of expression of hCerberus detected in these tissues.

TABLE 1

<u>TISSUE</u>	<u>Relative Level of Expression of hCER</u>
25 heart	undetectable
brain	undetectable
placenta	undetectable
lung	undetectable
liver	undetectable
30 skeletal muscle	undetectable
kidney	undetectable
pancreas	undetectable

TABLE 1 (cont'd)

<u>TISSUE</u>	<u>Relative Level of Expression of hCER</u>
spleen	undetectable
thymus	undetectable
5 prostate	undetectable
testis	undetectable
ovary	undetectable
small intestine	medium
colon (mucosa lining)	low
10 peripheral blood leukocytes	undetectable
stomach	high
thyroid	very low
spinal chord	very low
lymph node	medium
15 trachea	undetectable
adrenal gland	undetectable
bone marrow	undetectable
skeletal (muscle only)	undetectable
20 uterus (no endometrium) (muscle only)	low
colon (no mucosa) (muscle only)	very low
25 small intestine (muscle only)	high (highest of all tissues screened)
bladder (muscle only)	undetectable
30 heart (muscle only)	undetectable
stomach (muscle only)	high
prostate (muscle only)	undetectable

References:

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Furuta, Y., Piston, D. W., and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* 124, 2203-12.

Hemmati-Brivanlou, A., Kelly, O. G., and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* 77, 283-95.

Piccolo, S., Sasai, Y., Lu, B., and De, R. E. M. (1996). Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* 86, 589-598.

Smith, W. C., and Harland, R. M. (1992). Expression cloning of noggin a new dorsalizing factor localized to the spemann organizer in xenopus embryos. *Cell* 70, 829-840.

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Zimmerman, L. B., Jesus, E. J. M. D., and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86, 599-606.

25

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto

30 without departing from the spirit or scope of the appended claims.

Human cerberus nucleotide sequence - SEQ ID No. 1

10 20 30 40 50 60
 ATG CAT CTC CTC TTA TTT CAG CTG CTG GTA CTC CTG CCT CTA GGA AAG ACC ACA CGG CAC CAG GAT
 70 80 90 100 110 120 130
 GCC CGC CAG AAT CAG AGT TCT CTT TCC CCC GTA CTC CTG CCA AGG AAT CAA AGA GAG CTT CCC ACA
 140 150 160 170 180 190
 GGC AAC CAT GAG GAA GCT GAG GAG AAG CCA GAT CTG TTT GTC GCA GTG CCA CAC CTT GTA GCC ACC
 200 210 220 230 240 250 260
 AGC CCT GCA GGG GAA GGC CAG AGG CAG AGA GAG AAG ATG CTG TCC AGA TTT GGC AGG TTC TGG AAG
 270 280 290 300 310 320 330
 AAG CCT GAG AGA GAA ATG CAT CCA TCC AGG GAC TCA GAT AGT GAG CCC TTC CCA CCT GGG ACC CAG
 340 350 360 370 380 390
 TCC CTC ATC CAG CCG ATA GAT GGA ATG AAA ATG GAG AAA TCT CCT CTT CGG GAA GAA GCC AAG AAA
 400 410 420 430 440 450 460
 TTC TGG CAC CAC TTC ATG TTC AGA AAA ACT CCG GCT TCT CAG GGG GTC ATC TTG CCC ATC AAA AGC
 470 480 490 500 510 520
 CAT GAA GTC CAT TGG GAG ACC TGC AGG ACA GTG CCC TTC AGC CAG ACT ATA ACC CAC GAA GGC TGT
 530 540 550 560 570 580 590
 GAA AAA GTC GTT GTT CAG AAC AAC CTT TGC TTT GGG AAA TGC GGG TCT GTT CAT TTT CCT GGA GCC
 600 610 620 630 640 650 660
 GCG CAG CAC TCC CAT ACC TCC TGC TCT CAC TGT TTG CCT GCC AAG TTC ACC ACG ATG CAC TTG CCA
 670 680 690 700 710 720
 CTG AAC TGC ACT GAA CTT TCC TCC GTG ATC AAG GTG GTG ATG CTG GTG GAG GAG TGC CAG TGC AAG
 730 740 750 760 770 780 790
 GTG AAG ACG GAG CAT GAA GAT GGA CAC ATC CTA CAT GCT GGC TCC CAG GAT TCC TTT ATC CCA GGA
 800
 GTT TCA GCT TGA

HUMAN CERBERUS amino acid sequence - SEQ ID No. 2.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule encoding human cerberus.
2. An isolated nucleic acid molecule according to claim 1, having a sequence selected from the group consisting of:
 - (a) the nucleotide sequence comprising the coding region of the human cerberus as set forth in SEQ NO. 1;
 - (b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes a molecule having the biological activity of the human cerberus; or
 - (c) a nucleotide sequence which, but for the degeneracy of the genetic code would hybridize to a nucleotide sequence of (a) or (b), and which encodes a molecule having the biological activity of the human cerberus.
3. A vector which comprises a nucleic acid molecule of claim 1 or 2.
4. A vector according to claim 3, wherein the nucleic acid molecule is operatively linked to an expression control sequence capable of directing its expression in a host cell.
5. A vector according to claim 3 or 4, which is a plasmid.
6. Isolated human cerberus protein, or a fragment or derivative thereof having the biological activity of human cerberus.
7. Isolated human cerberus protein, having the amino acid sequence as set forth in SEQ. NO. 2.
8. A host-vector system for the production of human cerberus which comprises a vector of claim 3 or 4, in a host cell.

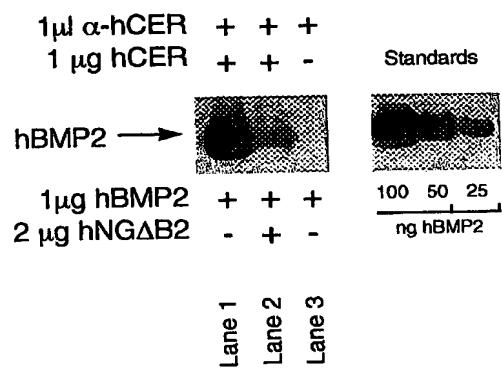
9. A host-vector system according to claim 8, wherein the host cell is a bacterial, yeast, insect or mammalian cell.
10. A method of producing human cerberus which comprises growing cells of a host-vector system of claim 8 or 9, under conditions permitting production of the human cerberus, and recovering the human cerberus so produced.
11. An antibody which specifically binds the human cerberus of claim 6 or 7.
12. An antibody according to claim 11, which is a monoclonal antibody.
13. An antibody according to claim 11, which is a polyclonal antibody.
14. A composition comprising human cerberus according to claim 6 or 7, and a carrier.
15. A composition comprising an antibody according to claim 11, 12, or 13 and a carrier.
16. Human cerberus according to claim 6 or 7, an antibody according to claim 11, 12, or 13 or a composition according to claim 14 or 15, for use in a method of treatment of the human or animal body, or in a method of diagnosis.
17. A polypeptide produced by the method of claim 10.
18. A ligandbody which comprises human cerberus fused to an immunoglobulin constant region.
19. The ligandbody of claim 18, wherein the immunoglobulin constant region

is the Fc portion of human IgG1.

20. A ligandbody according to claim 18 or 19, for use in a method of treatment of the human or animal body, or in a method of diagnosis.
21. A method of antagonizing the function of a Bone Morphogenic Protein (BMP) which comprises contacting said BMP with human Cerberus.
22. The method of claim 21, wherein the BMP is BMP2 or BMP4.
23. The method of claim 21 or 22, for use in treating BMP-related disorders of animals.
24. The method of claim 23, for treatment of a human.
25. The method of claim 24, wherein the BMP-related disorder is abnormal bone growth.
26. The method of claim 24, wherein the BMP-related disorder is Fibrodysplasia Ossificans Progressiva (FOP).
27. The method of claim 25, wherein the abnormal bone growth occurs following hip replacement surgery.
28. The method of claim 25, wherein the abnormal bone growth occurs following trauma, a burn or a spinal cord injury or in connection with metastatic prostate cancer or osteosarcoma.
29. A method of screening for a molecule capable of competing with human cerberus for binding to a BMP comprising:
 - a) contacting a sample suspected of containing the molecule with the BMP in the presence of human cerberus under conditions in which the human

cerberus is capable of binding to the BMP; and
b) detecting binding of the molecule to the BMP.

30. The assay of claim 29, wherein the BMP is BMP2 or BMP4.

Figure 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/06324

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/12 C07K14/475 C07K16/22 C12N15/62 A61K38/18		
According to International Patent Classification(IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DATABASE EMBL - EMEST13 Entry MMAA20122, Acc.No. AA120122, 21 November 1996 MARRA, M. ET AL.: "mn32d09.r1 Bedlington mouse embryonic region Mus musculus cDNA clone 538769 5'." XP002072013 cited in the application see the whole document</p> <p>---</p> <p style="text-align: center;">-/--</p>	1-10, 17
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input type="checkbox"/> Patent family members are listed in annex.
* Special categories of cited documents : <ul style="list-style-type: none"> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed 		
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search	Date of mailing of the international search report	
20 July 1998	30.07.98	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Smalt, R	

INTERNATIONAL SEARCH REPORT

Inte	onal Application No
PCT/US 98/06324	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOUWMEESTER T ET AL: "CERBERUS IS A HEAD-INDUCING SECRETED FACTOR EXPRESSED IN THE ANTERIOR ENDODERM OF SPEMANN'S ORGANIZER" NATURE, vol. 382, 15 August 1996, pages 595-601, XP002066227 cited in the application see the whole document ----	1-10,17
P,X	BIBEN, C. ET AL.: "Murine cerberus homologue mCer-1: A candidate anterior patterning molecule." DEVELOPMENTAL BIOLOGY, vol. 194, 15 February 1998, pages 135-151, XP002072011 See the whole document, particularly p. 139, left-hand column. ----	2-5,29, 30
P,X	BELO, J.A. ET AL.: "Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula." MECHANISMS OF DEVELOPMENT, vol. 68, November 1997, pages 45-57, XP002072012 see the whole document ----	2-5
A	LEMAIRE P ET AL: "The vertebrate organizer: structure and molecules" TRENDS IN GENETICS, vol. 12, no. 12, December 1996, page 525-531 XP004071057 see the whole document -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/06324

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.